

# Maintenance of gene silencing by the coordinate action of the H3K9 methyltransferase G9a/KMT1C and the H3K4 demethylase Jarid1a/KDM5A

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**Chromatin remodeling is essential for controlling the expression of genes during development. The histone-modifying enzyme G9a/KMT1C can act both as a coactivator and a corepressor of transcription. Here, we show that the dual function of G9a as a coactivator vs. a corepressor entails its association within two distinct protein complexes, one containing the coactivator Mediator and one containing the corepressor Jarid1a/KDM5A. Functionally, G9a is important in stabilizing the Mediator complex for gene activation, whereas its repressive function entails a coordinate action with the histone H3 lysine 4 (H3K4) demethylase Jarid1a for the maintenance of gene repression. The essential nature of cross-talk between the histone methyltransferase G9a and the demethylase Jarid1a is demonstrated on the embryonic *E $\gamma$ -globin* gene, where the concurrent introduction of repressive histone marks (dimethylated H3K9 and dimethylated H3K27) and removal of activating histone mark (trimethylated H3K4) is required for maintenance of gene silencing. Taken together with our previous demonstration of cross-talk between UTX and MLL2 to mediate activation of the adult  $\beta^{maj}$ -*globin* gene, these data suggest a model where “active” and “repressive” cross-talk between histone-modifying enzymes coexist on the same multigene locus and play a crucial role in the precise control of developmentally regulated gene expression.**

beta-globin locus | epigenetics | erythroid differentiation | hemoglobin

Histones are subjected to a number of posttranslational modifications that play important roles in regulating diverse cellular processes. These modifications are highly dynamic and are established through a competition between “writer” enzymes that introduce the modifications and “eraser” enzymes that remove them (1). Proper coordination between histone-modifying enzymes is critical for the regulation of gene expression (2, 3). For example, it has been shown that the histone H3 lysine 4 (H3K4) methyltransferases MLL3/4 (also called KMT2C/B) and the H3K27 demethylase UTX (also called KDM6A) are part of the same protein complex and coordinate their histone-modifying functions to activate the expression of specific genes through the concurrent introduction of the active histone mark trimethylated H3K4 (H3K4me3) and removal of the repressive histone mark H3K27me3 (4–6). These results underline the importance of cross-talk between histone methyltransferase and demethylase enzymes to activate transcription.

G9a (also called Ehmt2 or KMT1C) and its homolog GLP (also called Ehmt1 or KMT1D) have been identified as major euchromatic methyltransferases. These enzymes work as heterodimers to introduce monomethyl and dimethyl modifications on histone H3 at Lys-9 [single- or dimethylated H3K9 (H3K9me1/me2)] (7–10). In addition, several studies have shown that G9a and GLP mediate dimethylation of H3K27, both in vitro (11) and in vivo (12–14).

It has been well established that G9a can repress transcription by the introduction of repressive histone modifications (H3K9me2 and H3K27me2) and/or the recruitment of DNA methyltransferases (reviewed in 14, 15). In addition, G9a can activate transcription of

specific genes through a mechanism that does not require its methyltransferase activity (12, 16–19). It is currently unclear how G9a can act to mediate both the activation and repression of transcription. In addition, we do not know whether the G9a-GLP heterodimer interplays with other histone methylating/demethylating enzymes.

## Results

**G9a and GLP Associate with the Coactivator Complex Mediator and the Corepressor Protein Jarid1a.** To identify proteins that interact with the G9a-GLP heterodimer, we performed immunoprecipitation (IP) experiments using anti-GLP Abs and normal IgG as a negative control. Endogenous proteins were immunoprecipitated from nuclear extracts of murine erythroid (MEL) cells and identified by mass spectrometry (MS) (Fig. 1A and Dataset S1). Validating our approach, G9a and GLP themselves were identified, as well as a number of previously known G9a-interacting partners, including Wiz (20), DNMT1 (21), and HP1 $\gamma$  (22, 23). In addition, several previously unknown G9a-GLP interacting proteins, including both coactivators and corepressors, were identified, which is consistent with the dual role of G9a in regulating transcription (Fig. 1).

Among the coactivators that interact with G9a-GLP, 17 subunits of the Mediator complex, which is a fundamental component of the RNA polymerase II (Pol II) transcriptional machinery (24), were identified (Fig. 1). This result is consistent with our previous finding that G9a interacts with Pol II (12) and with the previously shown interaction between G9a and Med12 (25). Furthermore, we confirmed the association of G9a-GLP with the Mediator complex by both independent G9a and GLP IPs revealed by Western blot (Fig. 1B) and by a reciprocal Med1 IP in which Med12, G9a, and GLP were detected (Fig. 2B, Right).

In addition to the coactivator complex Mediator, several corepressor proteins were identified as G9a-GLP-associated partners, including the DNA methyltransferase DNMT1, the histone deacetylases HDAC1 and HDAC2, and HP1 $\gamma$ . Furthermore, the H3K4me3 demethylase Jarid1a (also called KDM5A or Rbp2) (26–28) was identified as a previously unknown G9a-GLP-interacting protein (Fig. 1).

In an attempt to separate the various corepressors that associate with G9a and GLP, we performed size exclusion chromatography on nuclear extracts. Consistent with G9a and GLP being part of a heterodimer (10), these two proteins coelute in the same two gel filtration fractions. Strikingly, the main peak of Jarid1a comigrates

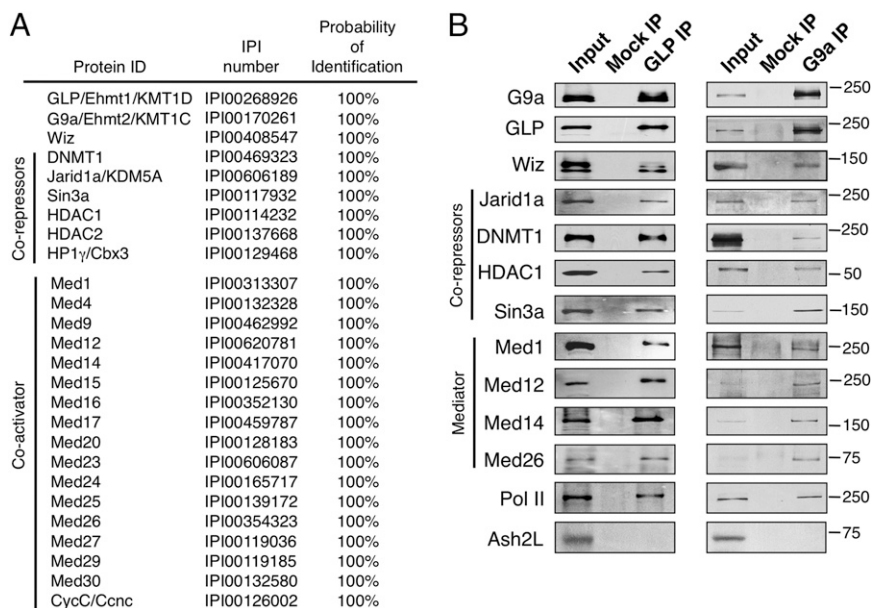
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**Fig. 1.** Identification of Jarid1a and the Mediator complex as previously undescribed G9a- and GLP-interacting proteins. (A) MS analysis of a GLP immunoprecipitate. The probability of identification was determined using ProteinProphet (44). IPI, International Protein Index. (B) Confirmation of the interaction of GLP and G9a with Jarid1a and the Mediator complex. Proteins immunoprecipitated via Abs against GLP and G9a were analyzed by Western blot. A mock IP with normal IgG was used as a negative control. Abs used for Western blot (Left) and molecular masses (Right; in kilodaltons) are indicated.

with the main peak of G9a and GLP (Fig. 2A, fractions 10 and 11). This is in contrast to other G9a-interacting proteins, such as DNMT1 (21), DNMT3a/b (29), and Wiz (20), or Jarid1a-interacting proteins Ezh2 and Suz12 (30, 31), which coelute with G9a and GLP but are present predominantly in lower molecular mass fractions (Fig. 2A, fractions 13–16). This result suggests that Jarid1a and the G9a-GLP heterodimer might be tightly associated in the cell. To address this possibility further, we performed a reciprocal IP using Abs against endogenous Jarid1a. We observed both G9a and GLP in the Jarid1a IP fraction, which confirms the interaction between these proteins (Fig. 2B). To determine whether the interaction between G9a and Jarid1a is direct, we incubated recombinant (rec) purified G9a and Jarid1a proteins and performed a G9a IP. As shown in Fig. 2C, rec G9a specifically pulled down rec Jarid1a (but not the control rec Ubc4), demonstrating a direct interaction between these proteins. Taken together, these results indicate that G9a, GLP, and Jarid1a are tightly associated within the same protein complex that is distinct from the core PRC2 complex containing Ezh2 and Suz12 (32).

Because these results were obtained using a mouse MEL cell line, we wanted to determine whether the interaction between G9a-GLP and Jarid1a is conserved in other cell types. To address this question, the G9a, GLP, and Jarid1a IPs, as well as the size exclusion chromatography experiments, were repeated with nuclear extract prepared from undifferentiated murine embryonic stem (mES) cells. The association between these proteins was reproduced in mES cells (Fig. S1), suggesting that the interaction between the G9a-GLP heterodimer and Jarid1a is conserved in cell types of different cell fate potential.

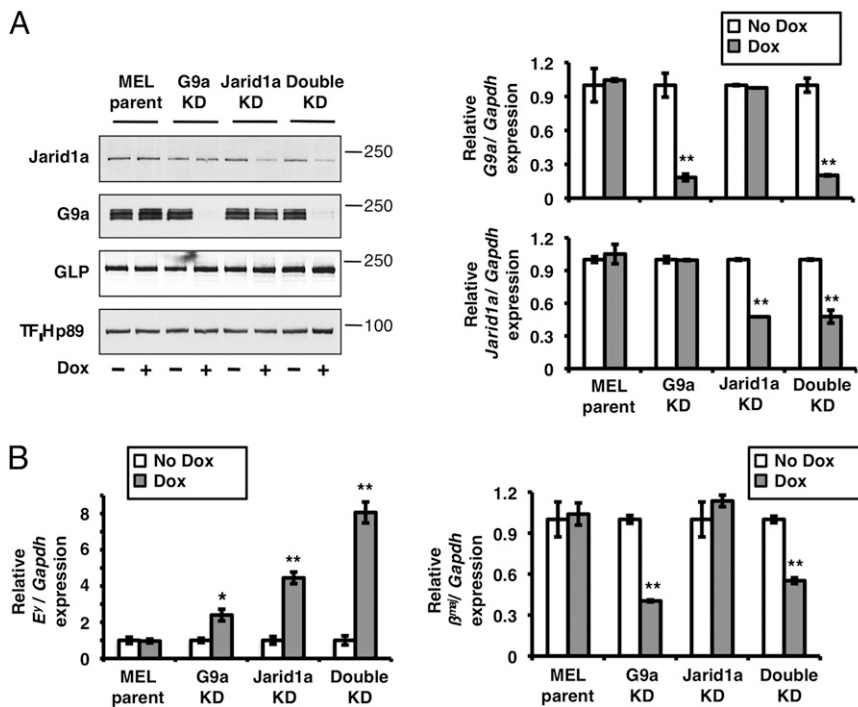
**Cross-Talk Between G9a and Jarid1a Is Important for the Maintenance of Gene Repression.** The interaction between G9a and Jarid1a is particularly interesting in light of the cross-talk between histone methyltransferase and demethylase that has been implicated in the regulation of transcription. Indeed, it has been shown previously that the interaction between the H3K4 methyltransferases MLL3/4 and the H3K27 demethylase UTX is important for the activation of transcription (4–6). In this context, the detected interaction between G9a and Jarid1a suggests that cross-talk between methyltransferase

and demethylase might also be important to maintain gene repression through a mechanism that involves the concurrent introduction of repressive histone marks (H3K9me2 and H3K27me2) by G9a and removal of active histone mark (H3K4me3) by Jarid1a. To test this possibility, we studied the mechanism of gene repression on the  $\beta$ -globin locus. This locus comprises several  $\beta$ -globin genes that are differentially regulated during development, including the embryonic gene  $E^y$ , which is repressed, and the adult gene  $\beta^{maj}$ , which is actively transcribed in differentiated MEL cells (33–36). Furthermore, it was shown previously that G9a maintains the  $E^y$  gene in a repressed state in a methyltransferase-dependent manner (12).

To determine whether Jarid1a is also involved in maintaining the  $E^y$  gene in a repressed state, stable MEL clones were generated in which the shRNA-mediated knockdown (KD) of Jarid1a can be induced on doxycycline (Dox) treatment. We found that although the KD of Jarid1a (Fig. 3A) does not affect the proliferation of cells during erythroid differentiation (Fig. S2), it leads to derepression of the embryonic  $E^y$  globin gene (Fig. 3B, Left), suggesting that Jarid1a is involved in maintaining this gene in a silenced state. In contrast, Jarid1a KD has no effect on transcription of the adult  $\beta^{maj}$  globin gene (Fig. 3B, Right). To determine whether Jarid1a directly represses  $E^y$ , the binding of Jarid1a to the promoter of this gene was analyzed by ChIP. Jarid1a was found to bind to the  $E^y$  gene promoter but not to the  $\beta^{maj}$  promoter or the distal enhancer HS2 in differentiated erythroid cells (Fig. 4A). In addition, Jarid1a binding was lost on Jarid1a KD (Fig. 4A), which validates the specificity of the Jarid1a Ab. Interestingly, Jarid1a binding to the  $E^y$  promoter is also decreased on the KD of G9a (Fig. 4B), emphasizing the importance of the interaction between G9a and Jarid1a for the binding of Jarid1a to chromatin. We note, however, that in the absence of G9a, Jarid1a binding to the  $E^y$  promoter is not completely lost (compare Fig. 4A and B). This residual weak binding could be due to the association of Jarid1a with other chromatin-associated proteins, such as GLP, which continues to interact with Jarid1a even in the absence of G9a (Fig. S3). Importantly, control experiments demonstrate that the KD of Jarid1a does not affect the amount of G9a (at the transcript and protein levels) and, similarly, that the KD of G9a does not affect the amount of Jarid1a in the cell (Fig. 3A). To determine whether



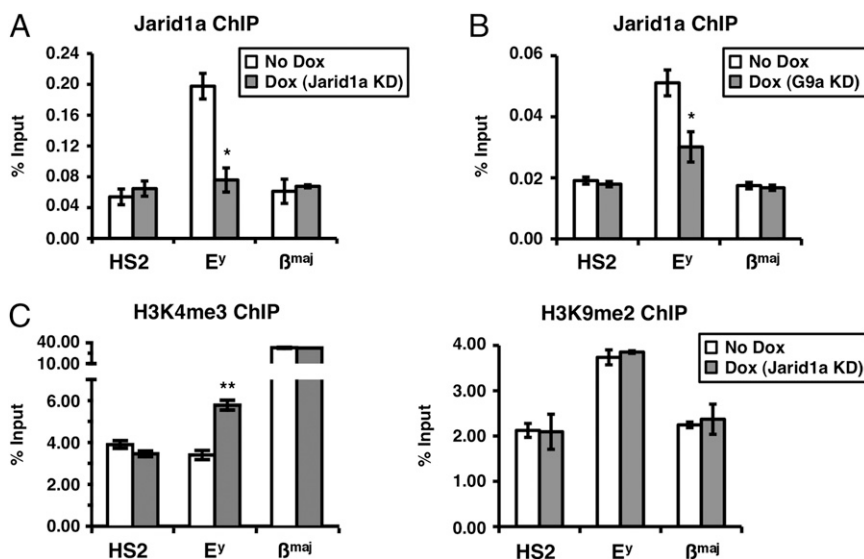




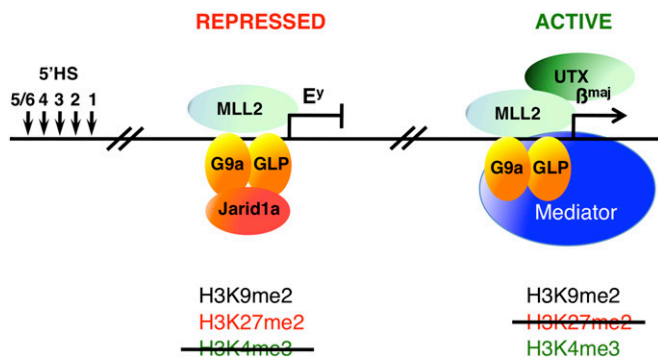
**Fig. 3.** Jarid1a and G9a cooperate in the maintenance of gene repression. (A) Establishment of stable MEL cell clones in which the single KDs of Jarid1a or G9a and the double KD of Jarid1a plus G9a are induced by Dox-mediated expression of specific shRNAs. This figure shows the results using Jarid1a shRNA1 (*SI Materials and Methods*). The levels of Jarid1a and G9a in Dox-treated (Dox) vs. untreated (No Dox) cells were analyzed at the protein level by Western blot of nuclear extracts using the indicated Abs (*Left*) and at the mRNA level by real-time RT-qPCR (*Right*). MEL parent represents a control cell line with no induction of shRNA on Dox treatment. (*Left*) Molecular masses of proteins (in kilodaltons) are indicated on the right. (*Right*) Transcript levels are normalized to *GAPDH* with the ratio observed in the absence of Dox set to 1. (B) Additive effect of the double Jarid1a plus G9a KD on derepression of the *E $\gamma$*  globin gene. Transcripts levels were measured by real-time RT-qPCR in Dox-treated/untreated cells. Transcript levels are normalized to *GAPDH* with the ratio observed in the absence of Dox set to 1. (A and B) Average values from triplicate experiments are represented with error bars corresponding to SDs. \* $P < 0.05$ ; \*\* $P < 0.01$ .

ase G9a and the demethylase Jarid1a. Furthermore, this mechanism may be generalized to additional Jarid proteins, because G9a is also present in a Jarid1c-containing complex (39).

**Cross-Talk Between Histone-Modifying Enzymes Differentially Regulates Gene Expression Within a Multigene Locus.** G9a plays a crucial role as a “gatekeeper” of cell identity by protecting cells from spurious



**Fig. 4.** Jarid1a maintains the globin gene *E $\gamma$*  in a repressed state through demethylation of H3K4me3. (A) Jarid1a binds to the *E $\gamma$*  promoter in erythroid cells. Jarid1a ChIPs were performed in the presence of normal (No Dox) or reduced (Dox) levels of Jarid1a. (B) G9a stabilizes the binding of Jarid1a to the *E $\gamma$*  promoter. Jarid1a ChIPs were performed in the presence of normal (No Dox) or reduced (Dox) levels of G9a. (C) KD of Jarid1a leads to an increase in H3K4me3 on the *E $\gamma$*  promoter. Native ChIPs were used to measure the levels of H3K4me3 and H3K9me2 in the presence of normal (No Dox) or reduced (Dox) levels of Jarid1a. (A–C) ChIPs were revealed by real-time qPCR using Taqman probes located at the HS2 site of the locus control region and at the promoters of the *E $\gamma$*  and  $\beta^{maj}$  genes. Error bars represent SDs calculated from triplicate experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ .



**Fig. 5.** Model of the regulation of transcription on the  $\beta$ -globin locus through cross-talk between histone methyltransferases and demethylases. In adult erythroid cells, the embryonic globin gene  $E^\gamma$  and the adult globin gene  $\beta^{maj}$  are both bound by the histone methyltransferases G9a-GLP, which introduce the repressive marks H3K9me2 and H3K27me2, and MLL2, which introduces the active mark H3K4me3. What differentiates these two genes is the presence of specific demethylases. Indeed, on the  $E^\gamma$  gene, the coordinate actions of the H3K4me3 demethylase Jarid1a and the H3K9/K27 methyltransferase G9a-GLP allow for the maintenance of a transcriptionally repressed state. In contrast, on the  $\beta^{maj}$  gene, the demethylase UTX actively removes the repressive mark H3K27me2, whereas the methyltransferase MLL2 trimethylates H3K4, allowing full activation of this gene in erythroid cells. 5'HS1-6 represents the distal  $\beta$ -globin locus control region.

expression of genes from other lineages (15). For example, G9a prevents the expression of neuronal genes in nonneuronal cell types (40) and the expression of pluripotency genes in differentiated cells (29). Similarly, G9a controls the developmental timing of gene expression within specific lineages, as exemplified by the derepression of neuronal progenitor genes in G9a-ablated adult neurons (40). In addition, G9a has a dual role in regulating transcription on the  $\beta$ -globin locus, where it is involved in both repressing the embryonic gene  $E^\gamma$  (through the introduction of repressive histone marks H3K9me2 and H3K27me2) and activating the adult gene  $\beta^{maj}$  (in a methyltransferase-independent manner) (12). However, it was not clear how G9a acts as both an activator and a repressor. The present study shows that the dual function of G9a entails an association with two physically distinct complexes: the coactivator Mediator and the corepressor Jarid1a. The existence of these distinct repressor and activator complexes is recapitulated on the  $\beta$ -globin locus, where the G9a-Mediator complex is bound to the active  $\beta^{maj}$  gene, whereas the G9a-Jarid1a complex is bound to the repressed  $E^\gamma$  gene (Fig. 5). On the  $\beta^{maj}$  promoter, G9a stabilizes binding of the Mediator complex (Fig. S5) to enable formation of the preinitiation complex and maximal transactivation of this gene (12) (Fig. 3B). In contrast, on the  $E^\gamma$  promoter, G9a is required for stable binding of Jarid1a (Fig. 4B) and for the maintenance of the  $E^\gamma$  gene in a repressed state through cross-talk with Jarid1a (Fig. 5). Future studies are required to determine what regulates the association of G9a with Jarid1a vs. Mediator.

Previously, it was shown that the H3K4 methyltransferase MLL2/KMT2D is bound to both the silenced gene  $E^\gamma$  and the active gene  $\beta^{maj}$  in differentiated erythroid cells (41). However, the MLL2-dependent H3K4me3 mark was detected on the  $\beta^{maj}$  gene but not on the  $E^\gamma$  gene. At this point, it was not clear whether MLL2 is enzymatically inactive on the  $E^\gamma$  gene or whether the H3K4me3 mark is dynamically removed. The finding here that Jarid1a demethylates H3K4me3 on the  $E^\gamma$  gene promoter suggests that MLL2 could be enzymatically active at this location but that the H3K4me3 mark is removed by Jarid1a. In contrast, the adult  $\beta^{maj}$  gene, which is highly enriched for H3K4me3, is not bound by Jarid1a. Instead, this gene is bound by another demethylase (i.e., UTX), which actively removes the G9a-dependent repressive mark H3K27me2, thereby allowing activation of the  $\beta^{maj}$  gene

(12). The presence of both MLL2 and UTX on the adult  $\beta^{maj}$  gene suggests that cross-talk between these histone-modifying enzymes is mediating activation of this gene. Therefore, there are at least two different examples of cross-talking between histone methyltransferases and demethylases that take place on the  $\beta$ -globin locus, one that activates transcription and one that represses transcription (Fig. 5, model).

Finally, it is interesting to note that although the repressed  $E^\gamma$  gene and the active  $\beta^{maj}$  gene are bound by both the histone methyltransferases MLL2 and G9a, the active vs. repressed status of these genes correlates with the presence of the histone demethylases (UTX vs. Jarid1a).

Taken together, these results reveal that “active” and “repressive” cross-talk of histone-modifying enzymes coexists on the same multigenic locus and plays a crucial role in the precise control of developmentally regulated gene expression.

## Materials and Methods

**Abs.** Abs used in this study are described in *SI Materials and Methods*.

**Nuclear Extraction and IPs.** Nuclear extraction and IPs were performed as described (41), except that proteins were eluted at 37 °C by 30 min of vortexing at 200  $\times$  g in a 6-M urea buffer containing 0.05% SDS, 50 mM Tris (pH 8.3), and 5 mM EDTA. Eluted proteins were analyzed by Western blot and/or digested with trypsin and analyzed by liquid chromatography (LC)-tandem MS (MS/MS). Before performing IPs for Western blot analysis, ethidium bromide was added to the nuclear extract at a final concentration of 100  $\mu$ g/mL.

**LC-MS/MS.** LC-MS/MS was performed on a linear trap quadrupole (LTQ) mass spectrometer with a nanospray ion source and Surveyor HPLC (Thermo). Data analysis was performed using the Trans-Proteomics Pipeline (TPP), version 4.0 (42), with SEQUEST and the International Protein Index mouse database (43). A probability of identification for each protein was determined using the ProteinProphet algorithm (44) integrated in the TPP.

**Size Exclusion Chromatography.** Nuclear extracts from MEL and mES cells were resolved by size exclusion chromatography using a Superose-6 10/300 GL column on an AKTA Explorer FPLC system following the instructions of the manufacturer (Amersham). Fractions of 0.5 mL were collected, precipitated with trichloroacetic acid (TCA), and analyzed by Western blot.

**MEL Clones with Inducible KD of Jarid1a and G9a.** MEL clones with Dox-inducible G9a KD were described previously (12). MEL clones with an inducible KD of Jarid1a and clones with inducible double KD of G9a plus Jarid1a were established as previously described (12). Details are provided in *SI Materials and Methods*. KD was induced by treating cells with 5  $\mu$ g/mL Dox for 4 d.

**ChIPs.** Histone ChIPs were performed using a native ChIP protocol (45). X-ChIPs were performed as described (41). Real-time quantitative PCR (qPCR) was done on a Rotorgene 6000 (Corbett Research) using TaqMan probes and primers. ChIP qPCR signals are presented as a percentage of input. Statistical significance was determined using a Student *t* test. Abs used for ChIP and primers/probes used for qPCR are discussed in *SI Materials and Methods*.

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